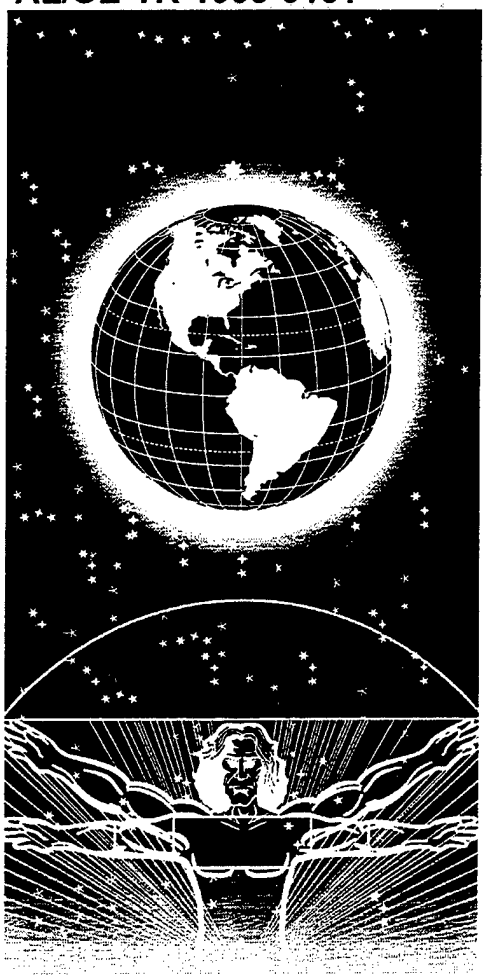
AL/OE-TR-1995-0161



UNITED STATES AIR FORCE ARMSTRONG LABORATORY

EFFECTS OF AMMONIUM DINITRAMIDE IN HUMAN LIVER SLICES: AN EPR/SPIN TRAPPING STUDY

L. Steel-Goodwin
K.W. Dean
OCCUPATIONAL AND ENVIRONMENTAL
HEALTH DIRECTORATE TOXICOLOGY DIVISION
ARMSTRONG LABORATORY
WRIGHT-PATTERSON AFB OH 45433-7400

D.M. Pace
NAVAL RESEARCH LABORATORY
WASHINGTON DC

A.J. Carmichael
ARMED FORCES RADIOBIOLOGY
RESEARCH INSTITUTE
BETHESDA MD

September 1995

Occupational and Environmental Health Directorate
Toxicology Division
2856 G Street
Wright-Patterson AFB OH 45433-7400

NOTICES

When US Government drawings, specifications or other data are used for any purpose other than a definitely related Government procurement operation, the Government thereby incurs no responsibility nor any obligation whatsoever, and the fact that the Government may have formulated, furnished, or in any way supplied the said drawings, specifications, or other data is not to be regarded by implication or otherwise, as in any manner licensing the holder or any other person or corporation, or conveying any rights or permission to manufacture, use, or sell any patented invention that may in any way be related thereto.

Please do not request copies of this report from the Air Force Armstrong Laboratory. Additional copies may be purchased from:

National Technical Information Service
5285 Port Royal Road
Springfield, Virginia 22161

Federal Government agencies and their contractors registered with the Defense Technical Information Center should direct requests for copies of this report to:

Defense Technical Information Service
8725 John J. Kingman Rd., Ste 0944
Ft. Belvoir, Virginia 22060-6218

DISCLAIMER

This Technical Report is published as received and has not
been edited by the Technical Editing Staff of the Air Force Armstrong Laboratory.

TECHNICAL REVIEW AND APPROVAL

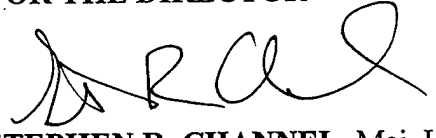
AL/OE-TR-1995-0161

The animal use described in this study was conducted in accordance with the principles stated in the "Guide for the Care and Use of Laboratory Animals", National Research Council, 1996, and the Animal Welfare Act of 1966, as amended.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE DIRECTOR



STEPHEN R. CHANNEL, Maj, USAF, BSC
Branch Chief, Operational Toxicology Branch
Air Force Armstrong Laboratory

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1995	Interim Report - December 1994-September 1995

4. TITLE AND SUBTITLE
Effects of Ammonium Dinitramide in Human Liver Slices: An EPR/Spin Trapping Study

5. FUNDING NUMBERS
Contract
PE 61102F
PR 2300
TA 2300OT
WU 2300OT51

6. AUTHOR(S)
L. Steel-Goodwin, K.W. Dean, A.J. Carmichael, and M.D. Pace

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Armstrong Laboratory, Occupational and Environmental Health Directorate
Toxicology Division, Human Systems Center
Air Force Materiel Command
Wright-Patterson AFB, OH 45433-7400

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Armstrong Laboratory, Occupational and Environmental Health Directorate
Toxicology Division, Human Systems Center
Air Force Materiel Command
Wright-Patterson AFB, OH 45433-7400

10. SPONSORING/MONITORING AGENCY REPORT NUMBER
AL/OE-TR-1995-0161

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Ammonium dinitramide (NH4N[NO2]2 ADN) is a high energy oxidizer currently under study by the US Air Force as a replacement for ammonium perchlorate (AP). ADN is believed to be more environmentally safe than AP. EPR/spin trapping studies with the spin trap N-tert-butyl-*a*-phenyl nitrone (PBN 10mM) for 5 min in the presence of ADN yielded an over lapping spin adduct EPR spectra. It consisted of a 1:2:2:1 quartet initially which was computer simulated using hyperfine coupling constants, an=ah=1.42 mT. After 60 min another spectra was also formed consisting of a triplet of doublets with hyperfine coupling constants an=1.65 mT and ah=0.4 mT. These radicals are similar to the effects of phosgene on PBN. Precision cut human liver slices were incubated with the spin trap PBN for 5 or 60 min with and without the 10 mM ammonium dinitramide (NH4N[NO2]2 ADN). The liver slices was homogenized, frozen in liquid nitrogen and lyophilized. ADN oxidized the PBN in human liver within 5 min exposure to form a yellow hydroscopic compound which was no longer paramagnetic. The reaction of ADN with liver caused acute injury.

14. SUBJECT TERMS
Ammonium dinitramide | Human | liver slices
Electron paramagnetic resonance EPR | spin trapping | N-tert-butyl-*a*-phenyl nitrone PBN

15. NUMBER OF PAGES
36

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

PREFACE

This is one of a series of technical reports generated from the experimental laboratory programs conducted in the Electron Paramagnetic Resonance laboratory for the Pharmacodynamic Group, Armstrong Laboratory, Toxicology Division. The research described in this report began in December 1994 and was completed in August 1995. It was sponsored by the Air Force Office of Scientific Research (AFOSR), Independent Laboratory Investigator Research Program, Work Unit # 2300OT51. Human liver was obtained from CPT Clay Miller, USA., under AFOSR Environmental Initiative Program Work Unit #2312A202. Lt Col Terry A. Childress served as Contract Technical Monitor for the U.S. Air Force, Armstrong Laboratory, Toxicology Division.

The authors gratefully acknowledge the following : SSGT W.W. Schmidt, SRA S Trivunovic and SSgt G. Miller for liver viability determinations and Mrs. Cressence Booher for secretarial assistance.

TABLE OF CONTENTS

LIST OF TABLES

TABLE **PAGE**

LIST OF FIGURES

FIGURES **PAGE**

LIST OF ABBREVIATIONS

ADN	**ammonium dinitramide**
ANOVA	**analysis of Variance**
AP	**ammonium perchlorate**
C	**centigrade**
dB	**decibel**
G	**Gauss**
h	**hour**
min	**minute**
mm	**millimeter**
mM	**millimoles/L**
mW	**milliwatt**
PBN	**N-tert-butyl-α-phenyl nitrone**
s	**second**
SCN–	**thiocyanate**
SD	**standard deviation**

INTRODUCTION

This is a study of the reactions of free radicals formed when ammonium dinitramide (ADN) is incubated with human liver slices. ADN is a high energy oxidizer and if used in solid fuel mixtures of projectiles could have propellant properties of importance to the Armed Forces. ADN is a candidate replacement compound for ammonium perchlorate (AP) in solid rocket fuel (Borman 1994). Solid rocket fuel is used to launch the Space Shuttle, the Delta II, Titan 34D, and Titan IV space vehicles. For the last thirty years AP has been an essential ingredient in the manufacture of this fuel. AP contains chlorine and forms radicals on decomposition (Hyde et al. 1961). It is therefore a potential environmental contaminant. ADN is being studied by the USAF as a replacement for AP, because ADN is less of an environmental hazard (Borman 1994).

The use of ADN as a propellant would involve exposure of humans, as well as the environment, to this chemical. Therefore, it is important to determine the effects of this exposure. In 1990 the Armstrong Laboratory was tasked by the United States Air Force to ascertain the toxic effects of ADN for occupational and environmental risk assessment. As a consequence, the following data has been gathered: ADN has an EC_{50} for proliferation of hepatocytes of 2.9 mM (Dean & Channel 1994), it is genotoxic as indicated by *in vitro* enzyme linked immunosorbant assay (ELISA) techniques (Dean & Channel 1994), and *in vivo* mouse micronuclei assays (Dodd 1994); ADN has no adverse effect on rabbit skin (Kinkead et al 1994a); ADN administered to rats by gavage has a calculated LD_{50} of 823 mg/kg (Kinkead et al 1994a) and ADN is a rat reproductive toxicant (Kinkead et al 1994b).

Chemical studies showed ADN decomposes to form free radicals (Steel-Goodwin et al 1994). One of the decomposition products of ADN is nitrogen dioxide (Pace 1994) and ADN on hydrolysis forms free radicals (Armengol 1994, Berty 1994, Young 1994).

The link between the free radical chemistry and the biological effects of ADN has been scarcely explored. Preliminary studies using WB344 rat liver cells showed ADN: decreased growth factor receptors (Armengol 1994, Young 1994); increased enzyme leakage (Berty 1994); reduced cell adhesion and induced cell death (Steel-Goodwin and Dean 1995).

A free radical is an atom, molecule or compound with one or more unpaired electrons (Rice-Evans et al 1991). Unpaired electrons occur in paramagnetic materials, the most important being organic free radicals, complex molecules containing transition metals such as iron and manganese and radiation damaged substances.

These chemical species are highly electrophilic and therefore can attack sites of increased electron density such as nitrogen atoms in DNA, RNA and proteins, carbon-carbon double bonds in unsaturated fatty acids and phospholipids (Rice-Evans et al 1991). Free radicals can be directly detected by the technique of electron paramagnetic resonance spectroscopy, EPR (Mason 1984; Rice-Evans 1991; Knecht & Mason 1993).

Qualitative EPR studies are of interest to the military: carbon tetrachloride (Mason 1984), $^{10}B(\alpha, n)\ ^{7}Li$ reactions (Carmichael et al 1988), tributyltin (Rivera et al 1992), nitric oxide (Steel-Goodwin et al. 1992), phosgene (Arroyo et al 1993), γ-radiation and non adenergic/noncholinergic nerve agents (Steel-Goodwin and Carmichael 1994), sulfur mustard (Arroyo et al 1995). However, toxicology studies, which will be used to develop a risk

assessment model, require a quantitative method rather than a qualitative method to determine free radical effects.

This investigation used precision cut tissue slices from donated human liver. In recent years, precision cut liver slices has emerged as a valuable system for understanding the toxic effects of xenobiotics (Fisher et al 1995). This study was designed to determine the effect of time and exposure of ADN on the number of radicals detected.

METHODS AND MATERIALS

Chemicals

N-Tert-butyl-α phenyl nitrone (PBN) and 2,2,5,5,-tetramethyl-1-pyrolindinyl oxy-3-carboxamide (3-CAR) were purchased from Sigma and Aldrich respectively. All other chemicals were reagent grade (Steel-Goodwin et al 1994).

Collecting samples and Trapping Radicals

ADN can generate radicals by chemical reactions in water or when liver mitochondrial enzymes metabolize this chemical. Human donor liver slices were prepared as described previously (Pravecek et al 1994). The slices were incubated at 37 °C in Waymouth's media supplemented with 10% fetal bovine serum and 10 mM PBN ± 10mM ammonium dinitramide (ADN). PBN was used in this study as it is the least toxic of all the traps as established by liver viability assessment measurements (Steel-Goodwin et al. 1994). The concentration of ADN used was chosen because it induced cell death in WB344 cells determined by ELISA assays (Steel-Goodwin and Dean 1995). After 5 min and 60 min exposure the liver slices were homogenized. A 20 ul sample was immediately analyzed for radicals and the rest was frozen in liquid nitrogen and lyophilized for 18 h. Lyophilized samples were stored in a dessicator at ambient temperature protected from light until analyzed. It was very important to keep the samples dry because moisture increases molecular mobility in the samples and decreases spin adduct lifetime and EPR signal intensity, therefore, interfering with the analysis.

Apparatus

The EMS 104 EPR Analyzer (Bruker Instruments INC., Billerica, MS) was purchased as a routine analysis tool for samples from tissues exposed to chemicals believed to generate free

radicals in biological tissue. The spectrometer consists of a microwave bridge, a permanent magnet and a detector. It is linked to a microprocessor (XPS P90Dell) via a RS232 interface which can be used to start remote acquisition and data collection for post processing using the WIN-EPR Windows 3.0 program (Bruker Instruments, INC.). This analyzer can detect at least 2×10^{10} spins/10^{-4} T. The magnetic field of the analyzer is applied to the sample while it is simultaneously being irradiated with microwaves. The magnetic field has the effect of aligning the magnetic moments of the electrons within the sample along two directions each having a slightly different energy. Transitions between these two energies give rise to spectroscopic interactions. An EPR spectrum is the first derivative of the electron paramagnetic absorption spectrum measured as a function of the magnetic field (Rice-Evans et al 1991).

Free Radical Analysis

The calibration procedures for the analysis were a four step process consisting of the following:

(1) Reference material

(2) Instrument set-up

(3) Routine spectrometer performance checks

(4) Establishment of a calibration curve

The reference material was made of known amounts of 3-CAR in distilled water or 3-CAR lyophilized with homogenized human liver not exposed to chemicals or trap. All data was standardized with 3-CAR in bicarbonate analyzed using a Bruker EMS300E spectrometer (Steel-Goodwin et al. 1994)

The manufacturer's procedures for the set up and instrument calibration were followed (Bruker, Inc. 1992) with appropriate electromechanical adjustments. The parameters for the spectrometer used to measure the liquid and solid samples are given in Table I.

Parameters	Liquid Samples	Solid Samples
Power (mW)	25.06	25.06
Sweep Width (G)	60	100
Modulation (G)	1.6	4.02
Sweep Time (s)	167.77	10.49
Filter Time Constant (ms)	1310.72	20.48
Receiver Gain (dB)	55	55
Field Offset (G)	5	0
Number of Sweeps	1	20
Sample Height (mm)	0	15

Table I Instrumental parameters used for analysis of liquid and solid samples.

The mean peak-peak EPR signal and standard deviation for each set of standards were measured. The best fit analytical form (linear, polynomial and exponential) was used to determine the goodness of fit of the calibration curves, (Sigma Plot, Jandel Scientific).

Liver slices exposed to chemicals or control media, were measured using the same parameters used to establish the standard curve. Each analysis was performed four times and each sample was measured twice. All data was normalized for sample weight. To determine that

the area of the spectra changed proportionally to the peak-peak analysis, liquid and solid samples were analyzed by both methods and the results compared by regression analysis (Sigma Plot, Jandel Scientific).

Experimental Design Analysis

Analysis of Variance was performed using the Design Ease ® Experimental Design program (Stat-Ease Inc., Minneapolis, MN). A two factorial analysis was performed to make the best use of the small number of slices available (n=16). We studied the effects two experimental parameters: time and ADN concentration, had on radicals detected in human liver.

RESULTS

ADN decomposes in water yielding free radicals in the presence of the spin trap PBN. The spectrum, Figure 1A, is the experimental result after 5 min. The free radical EPR spectrum is initially a 1:2:2:1 quartet with hyperfine coupling constants of a_N = 1.42 mT and a^{β}_H = 1.42 mT (Figure 1B). The 1:2:2:1 quartet corresponds to the reduction product of the spin trap 2-methyl-2-nitrosopropane (MNP). Therefore, the reaction of ADN with PBN indicates that the tert-butyl portion of the PBN including the nitrone group has been split from the PBN molecule. This decomposition of PBN could originate from the direct strongly oxidizing properties of ADN or from a strongly oxidizing radical product of ADN reacting with PBN.

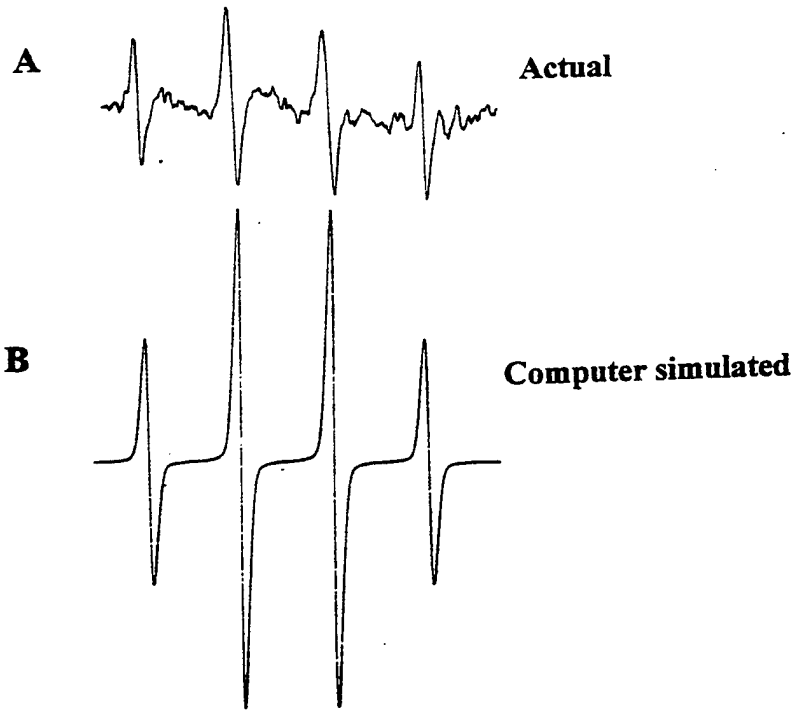


Figure 1 EPR spectrum 5 min. after hydrolysis of 10 mM ADN in 10 mM PBN.

After 60 min. the spectrum, Figure 2A, contains two radical species: a triplet of doublets with $a_N = 1.65$ mT and $a^{\beta}_H = 0.4$ mT (Figure 2B) and the initial 1:2:2:1 quartet, Figure 2C which combined gives spectrum, Figure 2D. It is possible that the triplet of doublets originates from the trapping of the aromatic portion of PBN by the tert-butyl-nitroso group after the decompostion of PBN. The longer time required to observe this adduct may be due to a low trapping efficiency which would require adduct build-up in order to be observed.

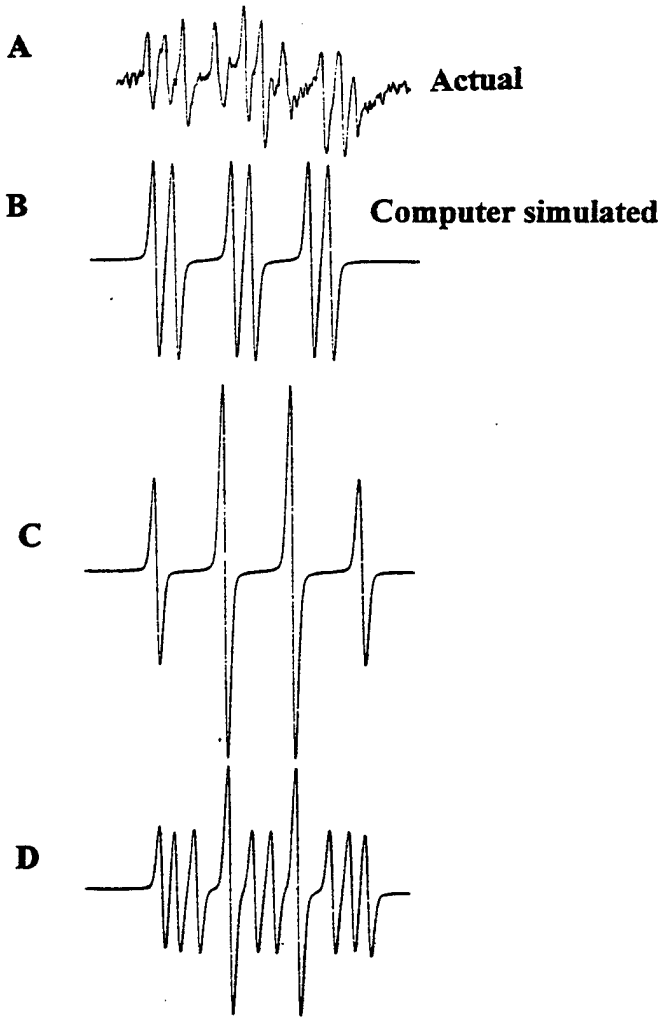


Figure 2 Spectrum 1hour after 10 mM ADN was hydrolyzed in 10 mM PBN.

To determine if time is a factor which will alter response of human liver to ADN exposure, we had to develop a method to quantitate radicals using the EPR analyzer. The stable radical 3-CAR was used to produce the calibration curve. The first derivative spectrum of 3-CAR dissolved in water is shown, Figure 3A. Peak-peak height was used to generate a calibration curve which was linear ($y = 1.0106x + 68.8221$ $r^2 = 0.99$), Figure 3B.

A **B**

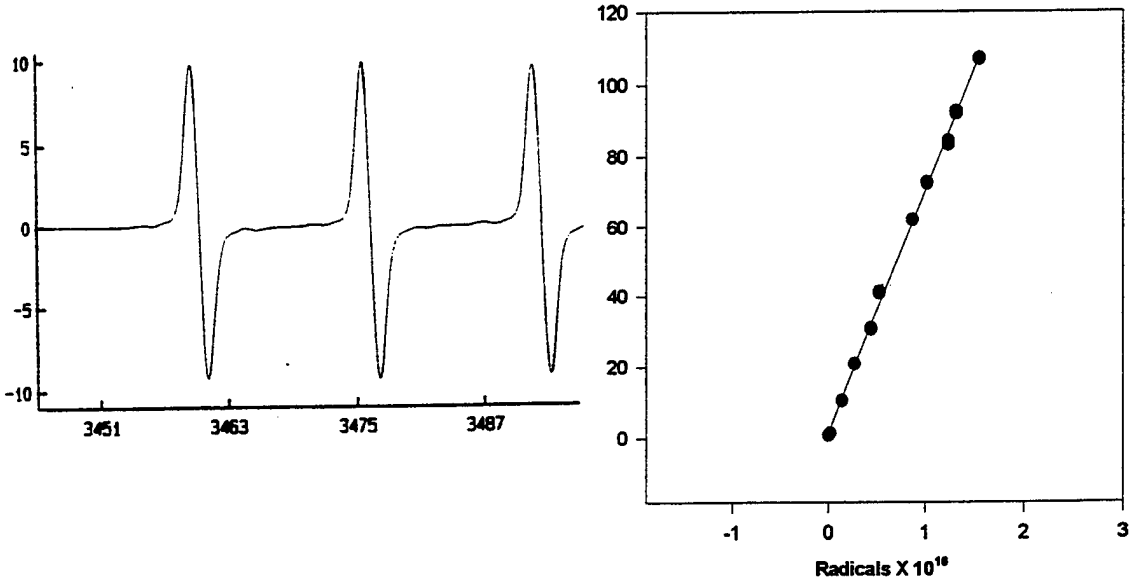


Figure 3 (A). Spectrum of 3-CAR. (B). Standard curve of peak height of 3-CAR.

When this experiment was repeated with human liver slices (20ul) there was no detectable signal in the liquid phase. Because we already saw a signal with ADN in water, we assumed antioxidants in the incubation media competed with the spin trap (10mM) for radicals. To

overcome this, we lyophilized the samples. Lyophilization still permits the trapped radicals to be detected by EPR without antioxidant interference.

The first derivative spectra of lyophilized human liver ± 10 mM ADN after 1h exposure is shown in Figure 4A & 4B respectively. There were radicals detected in the slices at both 5 and 60 min time points. However at 5 min. there was no difference in the radicals detected ± 10 mM ADN.

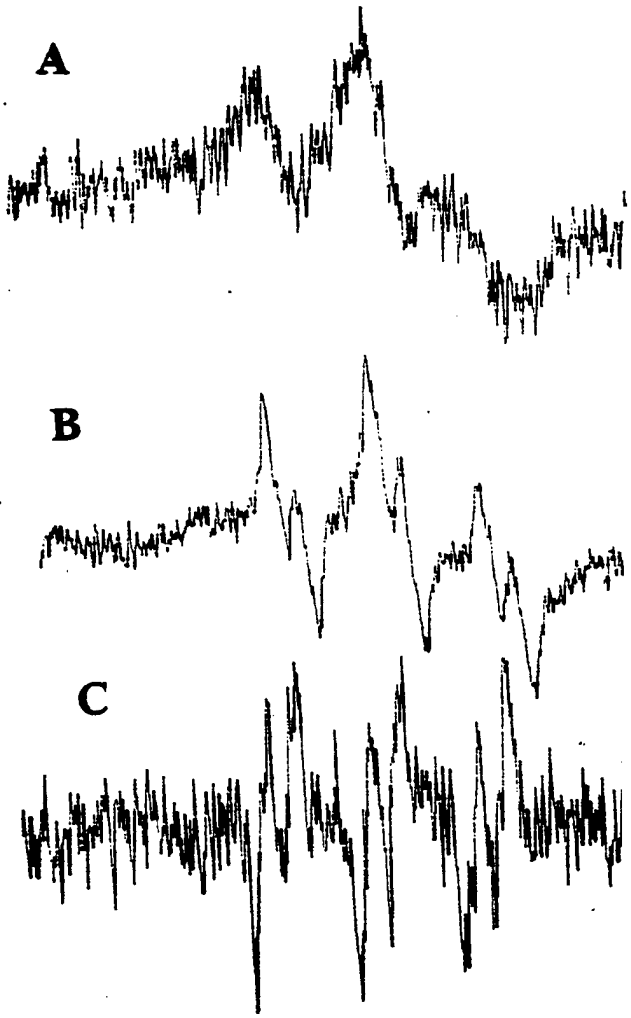


Figure 4 Spectrum of lyophilized human liver after one hour incubation: (A) Human liver exposed to 10 mM ADN.(B) Control liver incubated in 10 mM PBN. (C) Computer subtraction of A & B.

Radicals X 10^{17}

	5 min	60 min
Control	93 ± 6	333 ± 109
10 mM ADN	147 ± 183	37 ± 13

Table II. Mean ± SD (n = 4) of radicals measured in human liver slices exposed for 5 and 60 min to 0 or 10 mM ADN.

The statistical diagnosis and interpretation of the data in Table II is shown pictorially below, Figure 6.

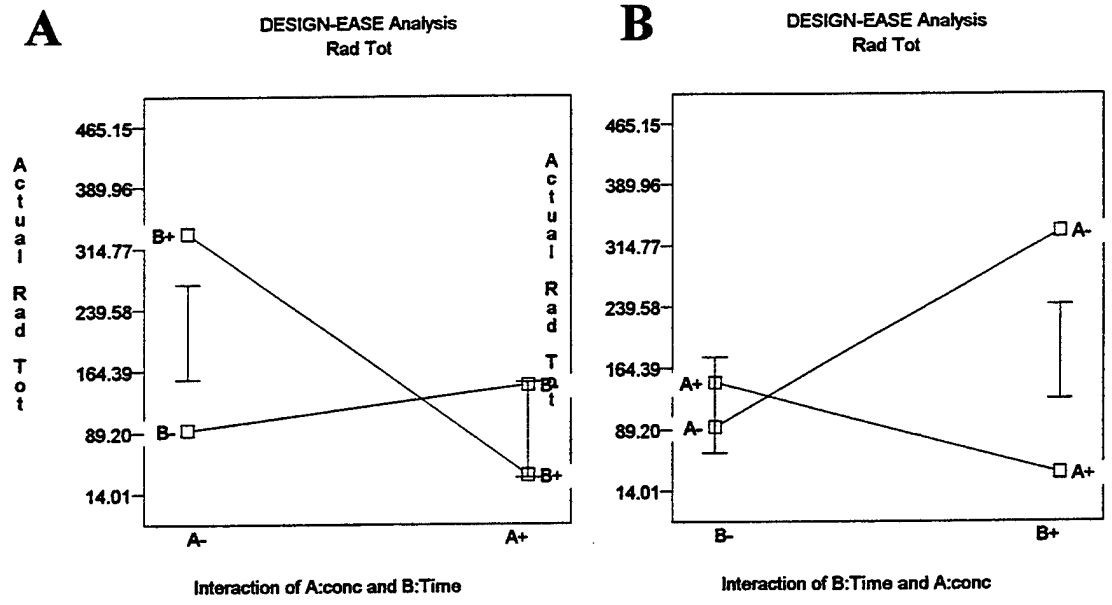


Figure 6 (A). Interaction of ADN of A: Concentration and B: Time with radicals. (B) Interaction of B: Time and A: Conc with radicals.

There was a difference in the radicals detected at the 60 min. time point ± 10 mM ADN. The computer subtraction of the radicals is shown in Figure 4C. As these liver samples were lyophilized, we prepared a standard curve of human liver lyophilized with increasing concentrations of 3-CAR. The first derivative spectrum of lyophilized 3-CAR in homogenized human liver is shown in Figure 5A. As the concentration of the 3-CAR decreases, there is a decrease in the peak heights.

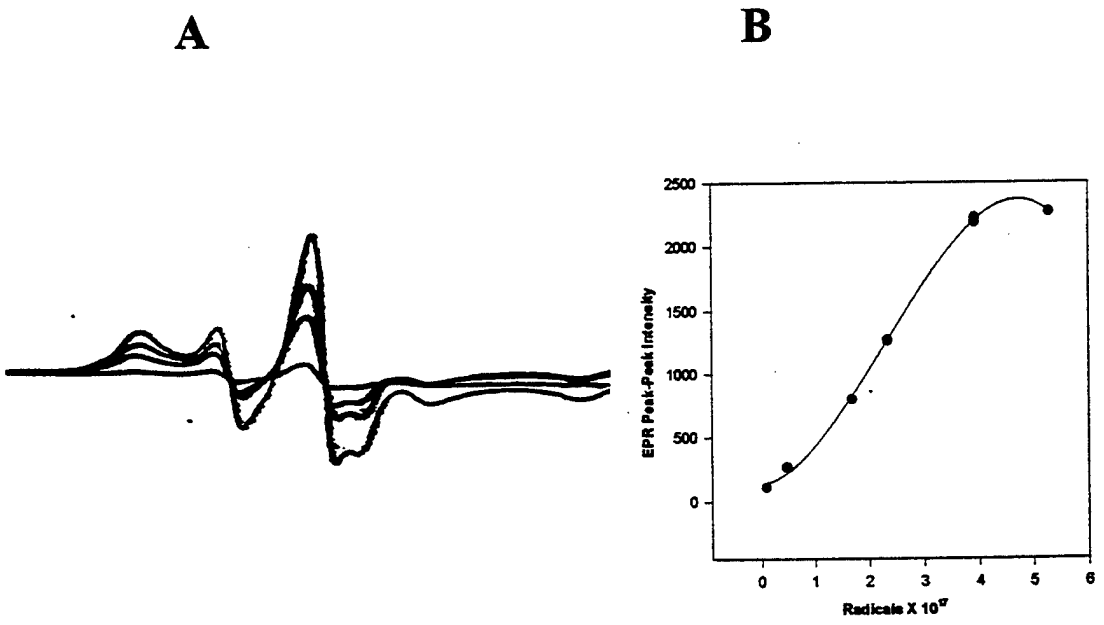


Figure 5 (A) Spectrum of lyophilized human liver incubated with 3-CAR. (B) Curve of peak height data of 3-CAR samples.

The peak-peak analysis curve was plotted (Figure 5B). This curve is linear from 0.1 to 4 X 10^{17} radicals ($y = 549.9242x - 6.1908$, $r^2 = 0.99$). Using the linear portion of the curve, the total number of radicals trapped by PBN when human liver is incubated at 5 min. and 60 min. were calculated, Table II.

The data had a normal distribution (results not shown). There were more radicals detected in the control liver in the first 5 min. than after 60 min. ($P< 0.05$), but there was no difference in the radicals measured in the ADN treated liver slices at either time point. The radicals generated by ADN in water were also quantitated over 5 min. and 60 min. There was ten times more radicals detected in the lyophilized tissue of human liver compared to the radicals detected in water alone. Liver contains paramagnetic material which could account for the difference.

All results reported were analyzed by peak-peak measurements. To show that the peak-peak measurements are proportional to the area under the spectrum, double integration of the data was performed on both liquid and solid samples. The equation of the plot of the liquid samples was $y = 1.5032\ x + 0.7653$, $r^2 = 0.99$ ($P< 0.001$). Figure 7 shows the data for solid samples and standards. The equation for this plot is $y= 1.0038\ x + 79.6401$ $r^2= 0.97$, ($P<0.05$).

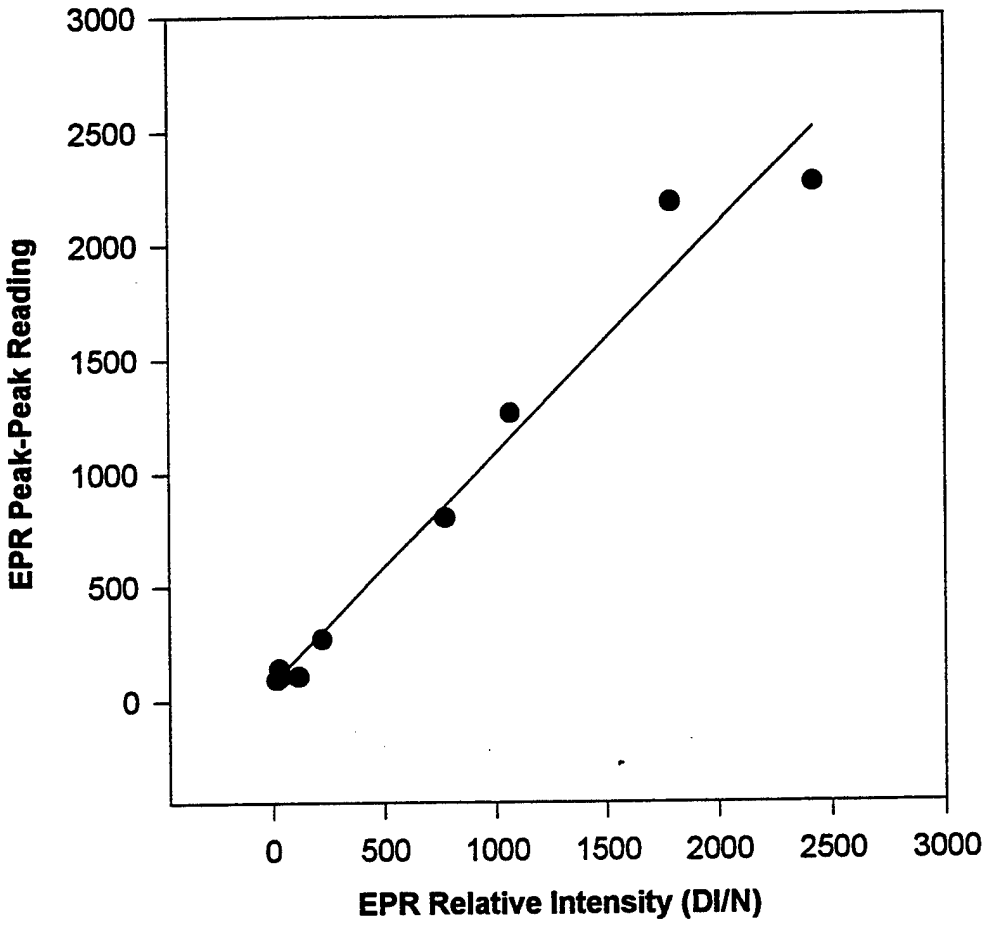


Figure 7 Comparison of peak-peak intensity and double integration of EPR signals of lyophilized liver samples.

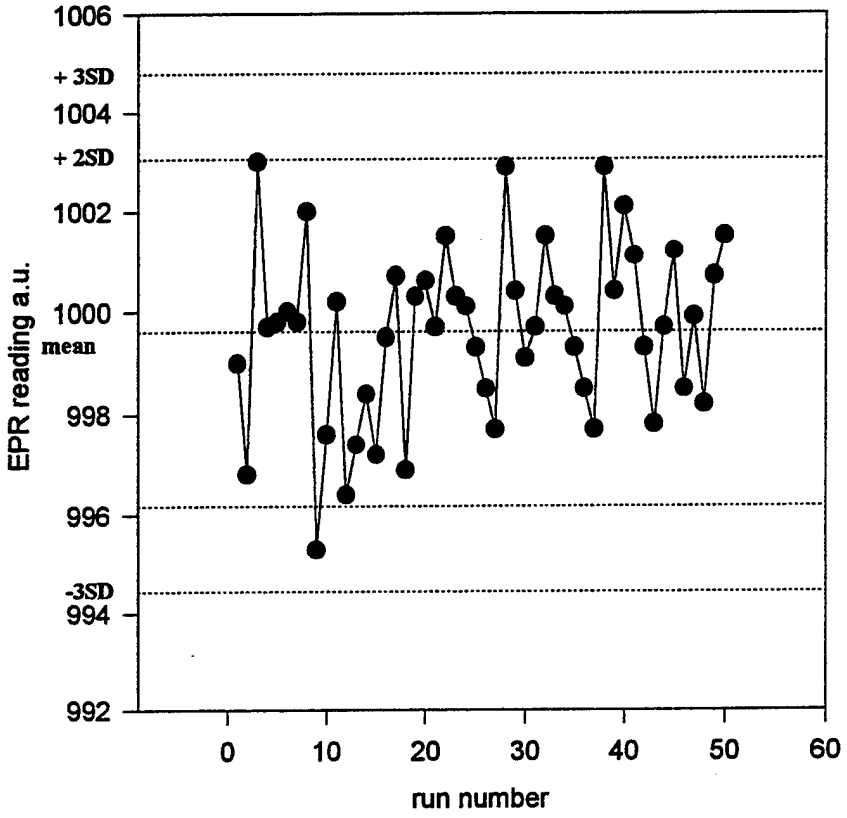


Figure 8 Plot of pitch calibrator on the days ADN project samples were analyzed.

Throughout this study, the EPR analyzer was in statistical control. This was determined by the plot of the EPR readings of the pitch used to calibrate the EPR instrument each day, Figure 8.

DISCUSSION

This investigation used precision cut tissue slices from donated human liver, to determine the radical effects of ADN. Human liver slices are a useful *in vitro* system, to investigate free radical effects of potential occupational and environmental chemicals.

EPR/spin trapping was used to detect radicals generated by 10 mM ADN in water and human liver samples. The radicals were quantitated with the 3-CAR as a standard. The spin trap PBN reacted with the radicals in the sample to form a more stable radical adduct which can be detected by EPR. Chemical radical reactions are very rapid. Radical identification by spin trap requires sufficient trap to be present, to compete with cellular components for the radicals (Steel-Goodwin and Carmichael 1995, Steel-Goodwin and Hutchens 1995). We were able to detect radicals generated by ADN and quantitate the number of radicals to which the human liver was exposed.

To quantitate radicals in this investigation, we calibrated the instrument using a pitch standard and used this to ensure the analyzer was providing a reading within statistical control, Figure 8. The concentration of radicals generated by 10 mM ADN was established using liquid and solid samples of the spin label 3-CAR, Figures 3 and 5 respectively. We found a linear correlation with concentration for the liquid and solid standards over the concentrations used for the standard curves. The homogenized liver media did not provide a detectable EPR signal in the liquid phase, but a signal was detected in the solid. The liver was exposed to 10 mM ADN for 5 or 60 min. The control liver sample at both time points was incubated only with trap.

Comparison of the spectra shows that there were differences in the radical species detected with time in the control species. These differences did not alter the quantitative data,

because the peak-peak and double integration of the data was proportional, Figure 7. Differences in radicals between controls and treated samples have been reviewed for carbon tetrachloride, (Mason 1984). Mason's review suggests that all EPR data should be assessed qualitatively as well as quantitatively.

The trapping efficiency of PBN for ADN is not known at this time, although the efficiency of PBN for the TCE radical and the spin label 3-CAR has been determined to be 20.6% and 31 % respectively (Steel-Goodwin and Carmichael 1995, Steel-Goodwin and Hutchens 1995). Therefore this data compares only the radicals trapped by the PBN.

Radicals are generated by ADN in water without liver. This suggests that ADN radical production does not require enzyme metabolism. EPR/spin trapping studies with the spin trap N-tert-butyl-α-phenyl nitrone (PBN 10mM) for 5 min in the presence of ADN yielded an over lapping spin adduct EPR spectra. It consisted of a 1:2:2:1 quartet initially which was computer simulated using hyperfine coupling constants, $a_N = a_H = 1.42$ mT. After 60 min another spectra was also formed consisting of a triplet of doublets with hyperfine coupling constants $a_N = 1.65$ mT and $a_H = 0.4$ mT. These radicals are similar to the effects of phosgene on PBN (Arroyo et al 1993). In these experiments the liver samples exposed to ADN were digested into a yellow liquid. This compound was very hydroscopic and the PBN-ADN reaction was no longer paramagnetic. It is predicted that a smaller concnetrations of ADN (<10mM) radicals generated by ADN will be sufficient to cause lipid peroxidation of the liver slices (Steel-Goodwin et al 1994). Lipid peroxidation is a chain reaction initiated when just a single radical removes an allylic hydrogen of a membrane polyunsaturated fatty acid, PUFA (Rice-Evans et al 1991). Reaction of this PUFA with oxygen forms a lipid hydroperoxyl radical, which can abstract an

studies (Steel-Goodwin et al 1994 and Pace 1994) and the radicals we detected with PBN, the following equations are proposed reactions which can occur on drinking ADN-H_2O:

1. $2NH_4^+N(NO_2)_2 \rightarrow 2NH_3\bullet + 4NO_2\bullet + 2H^+ + N_2$
2. $NH_3\bullet + H^+ \rightarrow NH_4^+$
3. $H_2O_2 + SCN^- + H^+ \rightarrow HOSCN + H_2O$
4. $RSH + OSCN^- \rightarrow RSOH + SCN^- + H^+$
5. $2H^+ + O_2^{-\bullet} + O_2^{-\bullet} \rightarrow H_2O_2 + O_2$
6. $2NO^\bullet + O_2 \rightarrow 2NO_2^\bullet$
7. $H_2O_2 + NO_2^- + H^+ \rightarrow ONOOH + H_2O$
8. $O_2^{-\bullet} + NO^\bullet + H^+ \rightarrow ONOOH$
9. $ONOOH \rightarrow {}^\bullet OH + NO_2^\bullet$
10. ${}^\bullet OH + NO_2^\bullet \rightarrow NO_3^- + H^+$
11. $O_2^{-\bullet} + Fe^{3+} \rightarrow O_2 + Fe^{2+}$
12. $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^\bullet OH + OH^-$
13. $NO_3^- + 3Fe^{2+} + 4H^+ \rightarrow NO^\bullet + 3Fe^{3+} + 2H_2O$
14. $H_2O_2 + O_2^{-\bullet} \rightarrow O_2 + OH^- + {}^\bullet OH$

ADN is very soluble in water (500g/L). ADN decomposes to form nitrogen dioxide ($NO_2^\bullet$) radicals in water and ammonium ions (Steel-Goodwin et al 1994, Pace 1994). If ADN-contaminated water is consumed, it will be swallowed with saliva. Human saliva has relatively high concentrations of peroxidase (H_2O_2) and thiocyanate (SCN^-), Grisham 1992. For example, levels of human saliva of non-smokers contains 0.1-0.3 mM SCN^- and 5 mM for smokers

allylic hydrogen from a neighbouring PUFA. This has the potential to begin a chain reaction to membrane destruction. The cell membrane contains many protein receptors which are involved in the regulation of cellular biochemistry. Hydroxyl radical-induced peroxyl radical formation by proteins causes peptide chain rupture which can ultimately lead to cell destruction.

Viable tissue produces a constant flux of free radicals such as superoxide, ($O_2^{\bullet-}$). The control samples were viable for the period of the study but the ADN treated samples were oxidized by the ADN within the first 5 min. of exposure.

Concurrent with this study, the technique has also been used to measure radicals *in vitro* in mouse liver slices exposed to cancer causing chemicals (Byczkowski 1994) and to quantitate radicals generated *in vivo* in B6C3F1 mice exposed to the carcinogen, trichloroethylene (Channel 1994). The results of these experiments are forthcoming. The EPR method does not destroy the sample, so it is more advantageous than fluorescence and other spectroscopic methods to study radicals. Also, the technology permits direct communication with other EPR spectrometers, located in other laboratories via computer link. This allows instantaneous verification of data by electronic means, as required, or simulation of spectra by other computer programs not available locally.

The EPR procedure used here was designed to demonstrate that EPR can be used to detect and quantitate radicals in liver samples exposed to chemicals, in particular, the effects of ADN exposure in human for risk assessment.

Human exposure to ADN through drinking water is currently highly remote but ADN is a potential hazard as the radicals generated by ADN occur on hydrolysis. Based on chemical

(Thomas et al 1991). Thus, ADN-water can react with SCN^- in the gut. Eosinophils found in the lamina propria of the gut wall are also sources of SCN^-. Exposure to ADN therefore has the potential to cause injury to mammalian cells by reaction with thiocyanate ions and peroxynitrite ions.

Our experiments have shown that the radicals generated by ADN do change with time. Overall, equations 1-14 suggest that the radicals generated by ADN decomposition will result in nitrates and nitrites. Thus, ADN dissolved in ground water may eventually become a substrate for microrganisms found in soil. Further studies using techniques to measure thiocynate, nitrites and nitrates *in vivo* after exposure to ADN could confirm these suggestions.

REFERENCES

Armengol, J.M. 1994 Effects of ammonium dinitramide on lactoferrin and transferrin receptors in liver: An EPR study. AFRRI/George Washington DoD Apprenticeship Program Report. Mentor: A.J. Carmichael

Arroyo, C.M., F. Feliciano, D.L. Kolb, J.R. Keeler, S.R. Millette and R.R. Stotts. 1993 Autoionization reaction of phosgene ($OCCl_2$) studied by EPR. J. Biochem Toxicology 8 (2) 107

Arroyo, C.M., R.L. von Tersch, C.A. Broomfield. 1995 Activation of alpha-human tumour necrosis factor (TNF-α) by Human monocytes (THP-1) exposed to 2-chloroethyl ethyl sulphide (HMG). Human and Experimental Toxicology 14 (7) 547.

Berty, S. 1994 The biological effects of ammoniumdinitramide on hepatocytes: An EPR study. Armstrong Laboratory and AFOSR Apprentice Report Mentor: Linda Steel-Goodwin.

Borman, S. 1994 Advanced energetic materials emerge for military and space applications. Chem & Engineering News 72: 18-22

Bruker Instruments Inc. 1992 EMS104 EPR analyzer's user's handbook.

Byczkowski, J.Z. 1994 Pharmacodynamic description of biologic effects: Chemically-induced oxidative stress. ManTech Environmental Technology Inc. Study Request F39.

Carmichael, A.J., C.M. Arroyo, M.L. Moore 1988 ESR and spin trapping of free radicals produced in poly U and poly A using the boron-neutron capture reaction. Radiat Phys Chem 32 (2) 227-231.

Channel, S.R. 1994 Trichloroethylene: Free radical production, oxidant damage and cell proliferation in B6C3F1 mouse liver. Work Unit 2312A202-Basic Environmental Research Initiative & 42230TO1-TCE Project.

Dean, K.W. and S.R. Channel 1994 *In vitro* effects of ammonium dinitramide AL/TR In Press.

Dodd, D. 1994 Genotoxicity assay of ammonium dinitramide *in vivo*: Micronuclei assay in mouse bone marrow cells. ManTech Environmental Technology, Inc Study# 1093-A06.

Kinkead, E.R., S.A. Salins, R.E. Wolfe, G.B. Marit 1994a Acute and subacute toxicity evaluation of ammonium dinitramide. AL/TR In Press.

Kinkead, E.R., R.E. Wolfe, C.D. Flemming, H.F. Leahy, D.J. Caldwell, C.R. Miller, and G.B. Marit 1994b Reproductive toxicity screen of ammonium dinitramide administered in the drinking water of Sprague-Dawley rats. AL-TR-WRAIR-TR-1994-0015.

Fisher, R.L. S.J. Hasal, I.G. Sipes, A.J. Gandolfi and K. Brendel 1995 Comparative metabolism and toxicity of dichlorobenzenes in Sprague-Dawley, Fisher-344 and human liver slices Human and Experimental Toxicology 14 (5): 414-421

Grisham, M.B. 1992 Reactive metabolites of oxygen and nitrogen in biology and medicine. CRC Press, Boca Raton, FL.

Hyde, J.S. and E.S. Freeman 1961 EPR observation of NH_3^+ formed by X-ray irradiation of ammonium perchlorate crystals. J. Chem 65: 1636-1638.

Knecht, K.T. and R.P. Mason 1993. *In vivo* spin trapping of xenobiotic free radical metabolites. Arch Biochemistry and Biophysics 303 (2) p185

Mason R.P. 1984 Spin trapping free radical metabolites of toxic chemicals. In Spin Labeling in Pharmacology. Chapter 2. Academic Press, Inc.

Pace, M.D. 1994 Spin trapping of nitrogen dioxide from photolysis of sodium nitrite, ammonium nitrate, ammonium dinitramide and cyclic nitramines. J Physical Chemistry 98: 6251

Pravecek, T.L., L. Steel-Goodwin, A.J. Carmichael, and S.R. Channel 1994 Free radical study using precision cut tissue slice techniques. J. Free Rad in Biol & Medicine Q15.

Rice-Evans,C.A., A.T. Diplock, M.C.R. Symons. 1991 Techniques in free radical research. ED. RH Burdon and PH van Knippenberg Elsevier, New York

Rivera J, S.C. Cummings., D.A. Macys 1992 *In Vitro* activation of lipophilic tributyltins by superoxide produces tributylstannyl superoxo radicals proposed initiateors of lipid peroxidation: An EPR model system Chem Res. Toxicol. 5: 698

Steel-Goodwin, L. and A.J. Carmichael 1994 Nitric oxide and smooth muscle relaxation in the intestine. Chemical and Radiation effects measured by EPR/spin trapping. In Radiation and the gastrointestinal tract. A. Dubois , G.L. King and D.R. Livengood Eds Chapter 7 CRC Press.

Steel-Goodwin, L. and A. J. Carmichael 1995 Trichloroethylene radicals: An EPR/spin trapping study Submitted AL/OLTechnical report

Steel-Goodwin, L. and K.W. Dean 1995 Apoptosis in liver cells on exposure to ammonium dinitramide. Ann Clin Lab Sci 25 (4) A33: 357-358.

Steel-Goodwin, L., B.H. Gray, C.M. Arroyo and A.J. Carmichael 1992 EPR detection of nitric oxide dependent spin adducts in mouse jejunum. In Biology of Nitric Oxide S. Moncada, M.A. Maletta, J.B. Hibbs Jr and E.a. Higgs Eds. Portaland Press London pp80-84.

Steel-Goodwin, L ., and B. Hutchens 1995 Efficiency of PBN to trap 3-CAR: An EPR Study. Submitted AL/OL Technical report.

Thomas, E.L., P.M. Bozeman, D.B. Learn 1991 Lactoperoxidase: Structure and catalytic properties. In: Peroxidases in Chemistry and Biology. J. Everse, K.E. Everse and MB Grisham Eds. CRC Press, Boca Raton, Ann Arbor, Boston, 124-138.

Young, A.J. 1994 Ammonium dinitramide effects on WB344 cell membranes: An EPR Study AFRRI/George Washington DoD Apprenticeship Program Report. Mentor: A.J. Carmichael.